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### Non-aqueous extracts of *Curcuma mangga* rhizomes induced cell death in human colorectal adenocarcinoma cell line (HT29) via induction of apoptosis and cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase

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#### ABSTRACT

**Objective:** To investigate the cytotoxic activity of the hexane and ethyl acetate extracts of *Curcuma mangga* rhizomes against human colorectal adenocarcinoma cell lines (HT29).

**Methods:** The cytotoxic activity of the hexane and ethyl acetate extracts of *Curcuma mangga* rhizomes against human colorectal adenocarcinoma cell lines (HT29) was determined by using the SRB assay.

**Results:** The ethyl acetate extract showed a higher cytotoxic effect compared to the hexane extract. Morphological changes of the HT29 cells such as cell shrinkage, membrane blebbing and formation of apoptotic bodies while changes in nuclear morphology like chromatin condensation and nuclear fragmentation were observed. Further evidence of apoptosis in HT29 cells was further supported by the externalization of phosphatidylserine which indicate early sign of apoptosis.

**Conclusions:** The early sign of apoptosis is consistent with the cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> checkpoint which suggests that the changes on the cell cycle lead to the induction of apoptosis in HT29.

## 1. Introduction

Colorectal cancer (CRC) can be defined as one of the cancer where it begins either in the colon or in rectum. CRC is commonly found on the epithelial lining of the large intestine or rectum. More than 95% of CRCs are adenocarcinomas. The origin of CRC is from the epithelial lining of the gastrointestinal tract and it is formed when normal colon/rectum cell undergoes a sequential of mutation on specific genetic material that altered the normal cell proliferation mechanism [1]. CRC is a common type cancer which was accounted for ten percent of all cancer in worldwide in year 2008 and 2012 [2]. In Peninsular Malaysia, The risk factors that influences the occurrences of CRC are included lifestyle habit, diet, genders, races and

genetic factors [3]. There are no early signs and symptoms of CRC during the early stages but the symptoms will be shown as the cancer grows in the late stage of CRC. The common symptoms are changes of bowel habit, rectal bleeding, stomach-ache, stomach cramping, weight loss and tiredness. The common treatments for CRC are chemotherapy, surgery, radiotherapy or combined treatments [4].

*Curcuma mangga* (*C. mangga*) is a species of rhizomes plant under the family of Zingiberaceae. It is locally known as 'temu pauh' in Malaysia while in Indonesia, it is known as 'temu mangga' or 'kunjir putih' and 'Khamin khao' in Thailand [5] because of the pleasant mango-scent rhizomes. Rhizomes and leaves of *C. mangga* had been reported to have cytotoxic activity and antiproliferation effects on breast adenocarcinoma, colorectal adenocarcinoma [6], oral carcinoma, lung adenocarcinoma, cervical carcinoma, colorectal carcinoma [7], ductal carcinoma [8,9], prostate carcinoma, lung carcinoma [10] and gastric adenocarcinoma [11,12]. Rhizomes and leaves extracts of *C. mangga* also possessed antioxidant [13–15], anti-inflammatory [16] and antimicrobial activities [17]. However, the mechanism of cell death on cancer cell induced by extracts of *C. mangga* has not been widely elucidated. Only one report by Karsono *et al.* (2014) demonstrated cell death through

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downregulation of the 5 $\alpha$ -reductase pathway of prostate cancer (PC)-3 induced by ethanolic extracts of *C. mangga* [18].

One of the hallmarks of cancer is resistance to programmed cell death or apoptosis [19]. Biochemical and morphological measures can be used to characterize apoptosis, such as pyknosis, chromatin condensation, and formation of apoptotic bodies [20], cell shrinkage [21], DNA fragmentation [22] and structure disruption of membrane [23]. In this communication, the induction of apoptosis of the hexane and ethyl acetate extracts of the rhizomes of *C. mangga* is reported.

## 2. Materials and methods

### 2.1. Plant material

The rhizomes of the *C. mangga* were obtained from Yogyakarta, Indonesia in July 2012. A voucher specimen (voucher number: HI 1331) was deposited in the Herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya.

### 2.2. Preparation of CME and CMH extracts

The hexane (CMH) and ethyl acetate (CME) extracts of *C. mangga* rhizomes were obtained according to the method of Malek *et al.* [7]. Briefly, the dried, ground and powdered rhizomes *C. mangga* (1.0 kg) was soaked in methanol (2.0 mL) for three days at room temperature and yielded 106.4 g of dark brown methanol extract (10.6%) after removal of the solvents using a rotary evaporator. The methanol extract was further fractionated consecutively with n-hexane, ethyl acetate and water to yield 35.7 g of hexane extract (33.6% of methanol extract), 30.1 g of dark brown ethyl acetate extract (28.3% of methanol extract) and 7.4 g of light brown water extract (7.0% of extract). CMH and CME extracts of *C. mangga* were prepared in dimethyl sulfoxide (DMSO) to obtain the 40.0 mg/mL stock solution for sulforhodamine B (SRB) cytotoxicity assay. A stock solution of both extracts was diluted to the required concentrations with Roswell Park Memorial Institute (RPMI 1640) medium to a final concentration of DMSO below 0.1% during the experiment.

### 2.3. Cell culture

Human colorectal adenocarcinoma cell lines (HT-29) and human normal colon cell lines (CCD-18Co) were purchased from American Type Culture Collection (ATCC, USA). Basic medium for culture of HT-29 and CCD-18Co cells was RPMI 1640 medium and minimum essential medium (MEM)

respectively. Both basic medium were supplemented with L-glutamine and other supplements such as fetal bovine serum, amphotericin B, penicillin/streptomycin, sodium pyruvate and non-essential amino acid solution. All basic mediums and supplements were purchased from Sigma–Aldrich, USA. HT-29 cells was cultured in RPMI 1640 medium supplemented with 10.0% fetal bovine serum, 2.0% of penicillin/streptomycin and 1.0% of amphotericin B while CCD-18Co was cultured in MEM medium supplemented with 20.0% fetal bovine serum, 2.0% of penicillin/streptomycin and 1.0% each for amphotericin B, sodium pyruvate and non-essential amino acid solution. All cells were grown in 25 cm<sup>3</sup> tissue culture flasks (Corning, USA). Both cell lines were incubated and maintained in incubator (Esco, Model: CCL-170B-8) under condition of humidified 5.0% CO<sub>2</sub> atmosphere at 37 °C. When the cell achieved 80%–90% confluent, it was detached by accutase and sub-cultured into new sterile culture flasks for further propagation.

### 2.4. SRB cytotoxicity assay

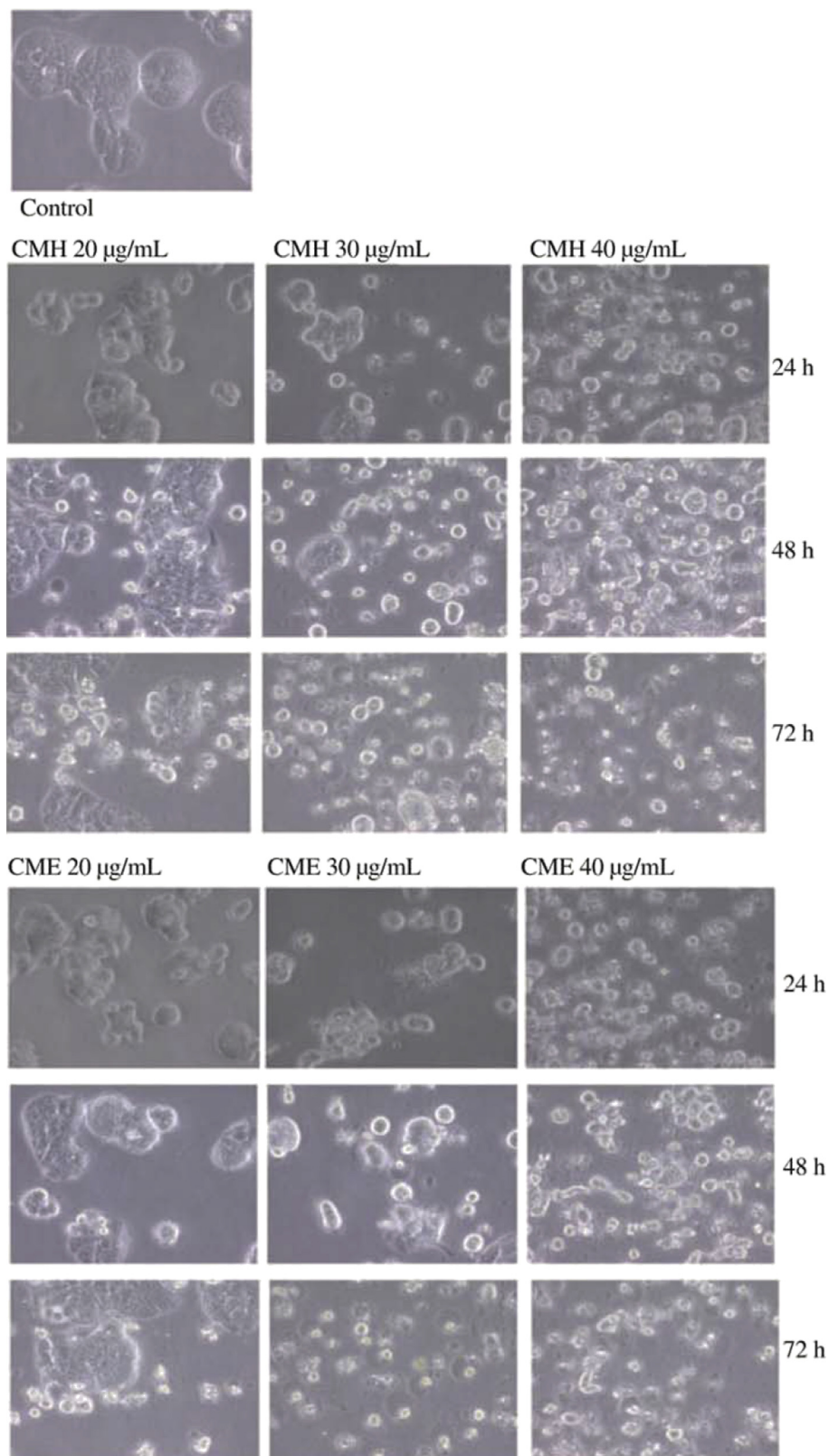
A flask of confluent cells was detached and cell at density of  $3.0 \times 10^4$  cells/mL were seeded onto 96-well plates and the seeded suspension cells attached in the wells after 24 h of incubation. After the incubation of 24 h, the media were discarded and stock solution of samples (CMH and CME) was diluted into various concentrations (ranging from 1.0  $\mu$ g/mL–100.0  $\mu$ g/mL) with the medium. The final concentration of DMSO in all wells did not exceed 0.1%. The treated cells were incubated for 24, 48 and 72 h at 37 °C in incubator. The untreated well was served as negative control. SRB assay was conducted as described by Houghton *et al.* (2007) with modifications. After the incubation period (24, 48 and 72 h), 50.0  $\mu$ L of 40.0% (v/v) trichloroacetic acid (TCA) was added to each well and kept at 4.0 °C for an hour. The medium containing TCA was discarded and the plates were added with 50.0  $\mu$ L of distilled water to remove the excess TCA in the wells. The washing process was repeated by replaced fresh distilled water for 4 times. Next, 50.0  $\mu$ L of 0.4% (w/v) SRB dye (Sigma) was added into well and the plates were incubate in room temperature for 30 min. The excess SRB dye was removed and washed the plates with 50.0  $\mu$ L of 1% (v/v) acetic acid for 5 times. 100.0  $\mu$ L of 10 mM of Tris Base was added in the plates and the plate was agitated for 500 rpm in the Thermo Shaker, BioSan, Latvia for 5 min to solubilise the bound SRB dye in every well. The absorbance reading was then calculated by using a microplate reader (Synergy H1 Hybrid) at wavelength 570 nm and 630 nm as background. The IC<sub>50</sub> was calculated by comparing the percentages of absorbance of treated cell with untreated cells (control) [24,25]. The assays were conducted in triplicates.

**Table 1**

IC<sub>50</sub> values of CME, CMH and cisplatin on HT29 and CCD-18Co.

Cell	Type	Time (h)	IC <sub>50</sub> ( $\mu$ g/mL)		
			CMH	CME	Cisplatin
HT29	Human colorectal adenocarcinoma	24	39.3 $\pm$ 6.0	32.2 $\pm$ 2.7	–
		48	21.7 $\pm$ 0.8	32.1 $\pm$ 2.6	–
		72	17.9 $\pm$ 1.2	15.6 $\pm$ 0.8	8.9 $\pm$ 1.2
CCD-18Co	Normal human colon	72	45.7 $\pm$ 1.0	46.5 $\pm$ 0.5	5.5 $\pm$ 0.5

Results are shown as mean  $\pm$  SE from three independent experiments.



**Figure 1.** Morphological changes of cells under phase-contrast microscopy at 40× magnification.

### 2.5. Morphological assessment

Phase contrast microscopy: Cells were seeded in sterile 24-well plate at a cell density of  $3.0 \times 10^4$  cells/mL and the cells were incubated for 24 h to allow adherence of cells. Then the cells were treated with both extracts at three concentrations

(20.0 µg/mL, 30.0 µg/mL and 40.0 µg/mL) and incubated for 24, 48 and 72 h in CO<sub>2</sub> incubator. The morphological changes of the treated cells included cell shrinkage; blebbing of membrane; cell detachment and rounding were observed. The image of morphological changes of the cells was captured at 40×

magnifications under phase contrast microscope (Zeiss Axio Vert, A1).

Fluorescence microscopy: Hoechst 33342/propidium iodide (PI) double staining method was used to observe the cell nuclear morphological changes in fluorescence microscopy [26]. The procedures in cell seeding and treatments were similar with the previous experiment with phase contrast microscopy. After the incubation period, the media in the plates were removed and the cells were harvested. Both media and harvested cells were collected and then centrifuged at 1500 rpm for 5 min. The cell pellets were washed with ice-cold phosphate buffer saline (PBS) solution and re-suspended with PBS solution. The cell suspensions were then incubated with Hoechst 33342 in the dark at 37 °C and humidified 5% CO<sub>2</sub> incubator for 7 min. Then PI was added into the cell suspension and further incubated in the dark under for 15 min at room temperature. Ices were used to deactivate and stabilize the activity of Hoechst dye. The cell samples were loaded on a slide and the morphology of cells was examined under Leica, Germany DM16000B fluorescent microscope. The image of nuclear morphological changes of the cells was captured at 40× magnifications.

## 2.6. Detection of early and late apoptosis through binding of Annexin V to phosphatidylserine

Detection of early and late apoptosis by binding of Annexin V to phosphatidylserine (PS) was analyzed using a flow cytometer. The overall cell apoptosis detection procedures were similar to Ho *et al.* (2013) with slight modification on the cell numbers [27]. Briefly,  $1.2 \times 10^5$  cells/mL was plated and treated with 3 different concentrations (20.0, 30.0 and 40.0 µg/mL) for 24, 48 and 72 h in triplicates. The cells were harvested into 15.0 mL centrifuge tubes and washed with PBS. Cell pellet

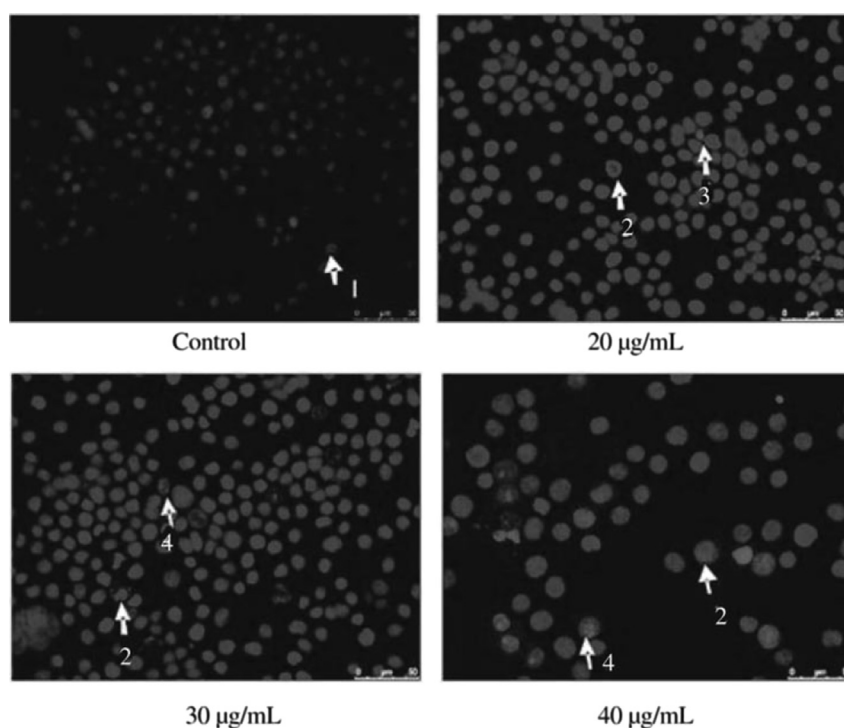
was re-suspended with Annexin-V binding buffer and transferred into 1.5 mL centrifuge tube. Suspended cells were stained with Annexin-V and propidium iodide for 15 min in the dark at room temperature. Annexin-V binding buffer was later added into stained cells to deactivate the staining process of Annexin-V and propidium iodide. Detection of apoptosis was performed using Accuri C6 flow cytometer. For each measurement, 10000 events were counted where cell population was distributed into different quadrants and each quadrant was analysed with quadrant statistics. Lower left quadrant represented viable cells; lower right quadrant represented early apoptotic cell; and upper right quadrant represented late apoptotic or necrotic cells.

## 2.7. Cell cycle analysis

The cell cycle analysis procedure was according to Ho *et al.* (2013) with slight modifications [27]. Briefly, HT29 cells were plated at cell number of  $3.0 \times 10^5$  cells/mL and treated with both extracts for 72 h in triplicates. The cells were harvested, washed and then fixed with 70% ethanol. The cell fixation took overnight time in −20 °C freezer. The next day, the fixed cells were centrifuged to become pellet, washed with ice-cold PBS and re-suspended with staining buffer containing 50 mg/mL of propidium iodide, 0.1% Triton-X-100, 0.1% sodium citrate, and 100 mg/mL of RNase. The cell suspension was incubated in the dark condition for 30 min at room temperature. The stained cell was then analyzed with Accuri C6 flow cytometer.

## 2.8. Statistical analysis

All results were expressed as mean ± SD and all the experiments were performed at least twice using sample



**Figure 2.** Nuclear morphology changes observed on HT29 treated with CMH after 72 h using double staining Hoechst 33342/PI at magnification of 40×. Arrows represent (1) Live cell with normal nuclei, (2) Live cell with apoptotic nuclei, (3) Dead cell with normal nuclei, and (4) dead cell with apoptotic nuclei.



triplicate. All raw data were statistically processed with IBM SPSS Statistics 22.0 software. *t*-test was performed to determine the significant differences between the treated cell and non-treated cell.  $P < 0.05$  was considered statistically significant.

### 3. Results

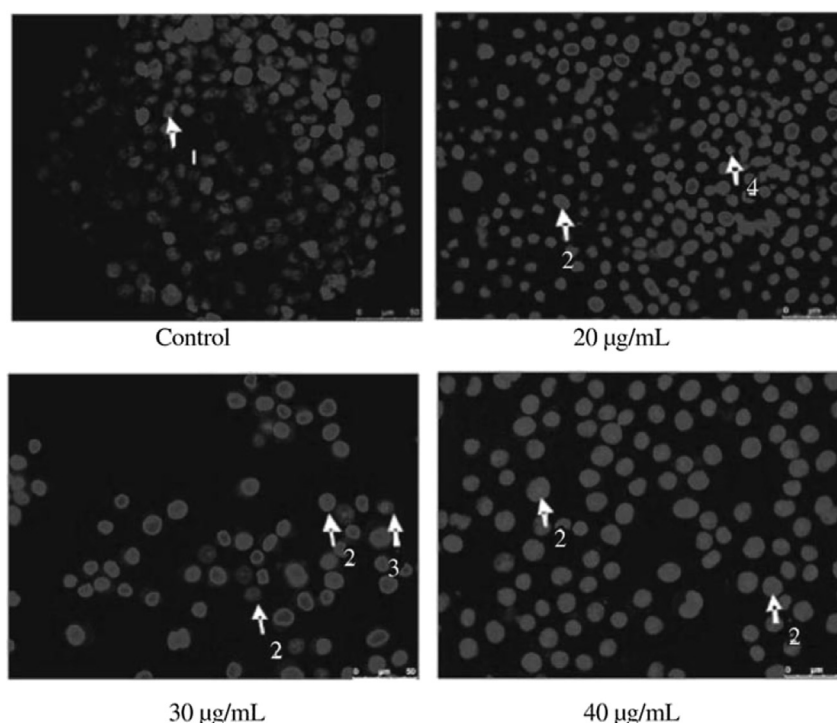
#### 3.1. Effects of CMH and CME extracts on cell growth inhibition of human colorectal adenocarcinoma (HT29) and normal human colon cell lines (CCD-18Co)

Results from SRB cytotoxicity assay showed that growth inhibitory effect against HT29 cells by both hexane and ethyl acetate extracts of *C. mangga* rhizomes increase in both dose- and time-dependent manner. Table 1 showed the half maximal inhibitory concentration (IC<sub>50</sub>) of CMH, CME and cisplatin (positive control) on HT29 and CCD-18Co cells at dose-dependent manner.

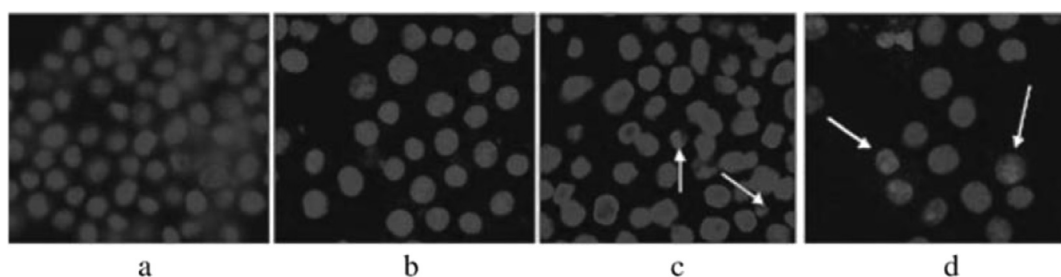
#### 3.2. CMH and CME extracts induced changes of cell morphology in HT29 cells

Phase contrast microscopy on HT29 cells showed the presence of floating or detachment of non-viable cells in dose- and time-dependent manners (Figure 1). Others morphological changes on treated HT29 cells were cell shrinkage, cell membrane blebbing and formation of apoptotic bodies. The detachment of non-viable cells and other morphological changes were similar in HT29 cells treated with both CMH and CME extracts.

The nuclear morphology changes in HT29 cells were observed through the fluorescence emissions from the nuclei of the cells after 72 h of treatment with both extracts. Nuclear morphological changes were observed by Hoechst 33342/PI double staining assay. Figures 2 and 3 shows viable cells in the negative control displays lower intensity blue fluorescence emission (Hoechst 33342 dye), where treated cells showed higher intensity of blue fluorescence emission and slight pink fluorescence (PI dye) in the nucleus. Figure 4 shows the nuclear morphology changes in a single cell.

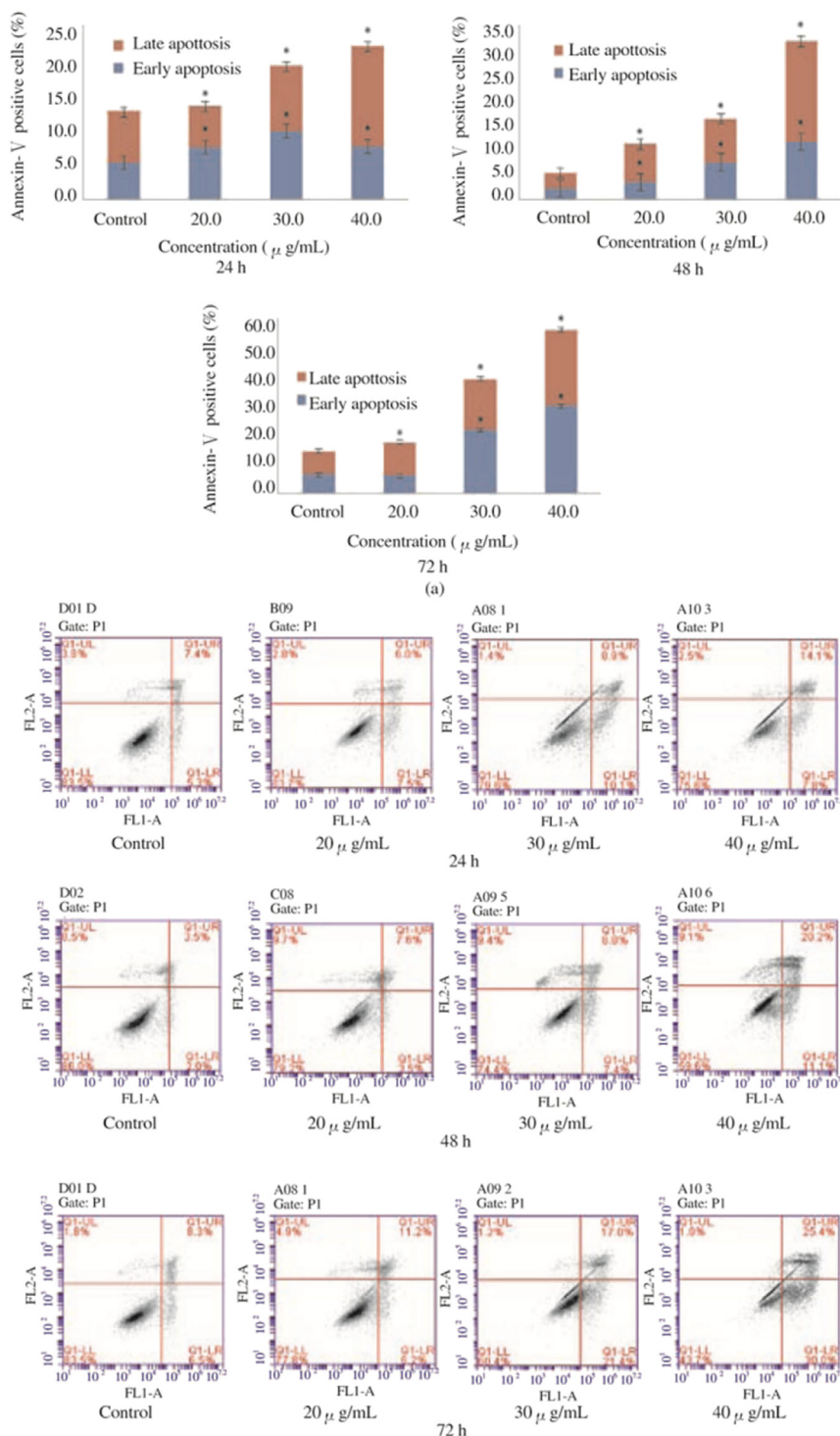


**Figure 3.** Nuclear morphology changes observed on HT29 treated with CME after 72 h using double staining of Hoechst 33342/PI at magnification of 40 $\times$ . Arrows represent (1) Live cell with normal nuclei, (2) Live cell with apoptotic nuclei, (3) Dead cell with normal nuclei, and (4) dead cell with apoptotic nuclei.



**Figure 4.** Nuclear morphology changes in a single cell.

(a) In negative control, most of the cells are viable with normal nuclei and lower intensity blue fluorescence emission (Hoechst 33342 dye). In treated cells, most of the cells undergo early apoptosis (b) and secondary necrosis (c) or late apoptosis (d).



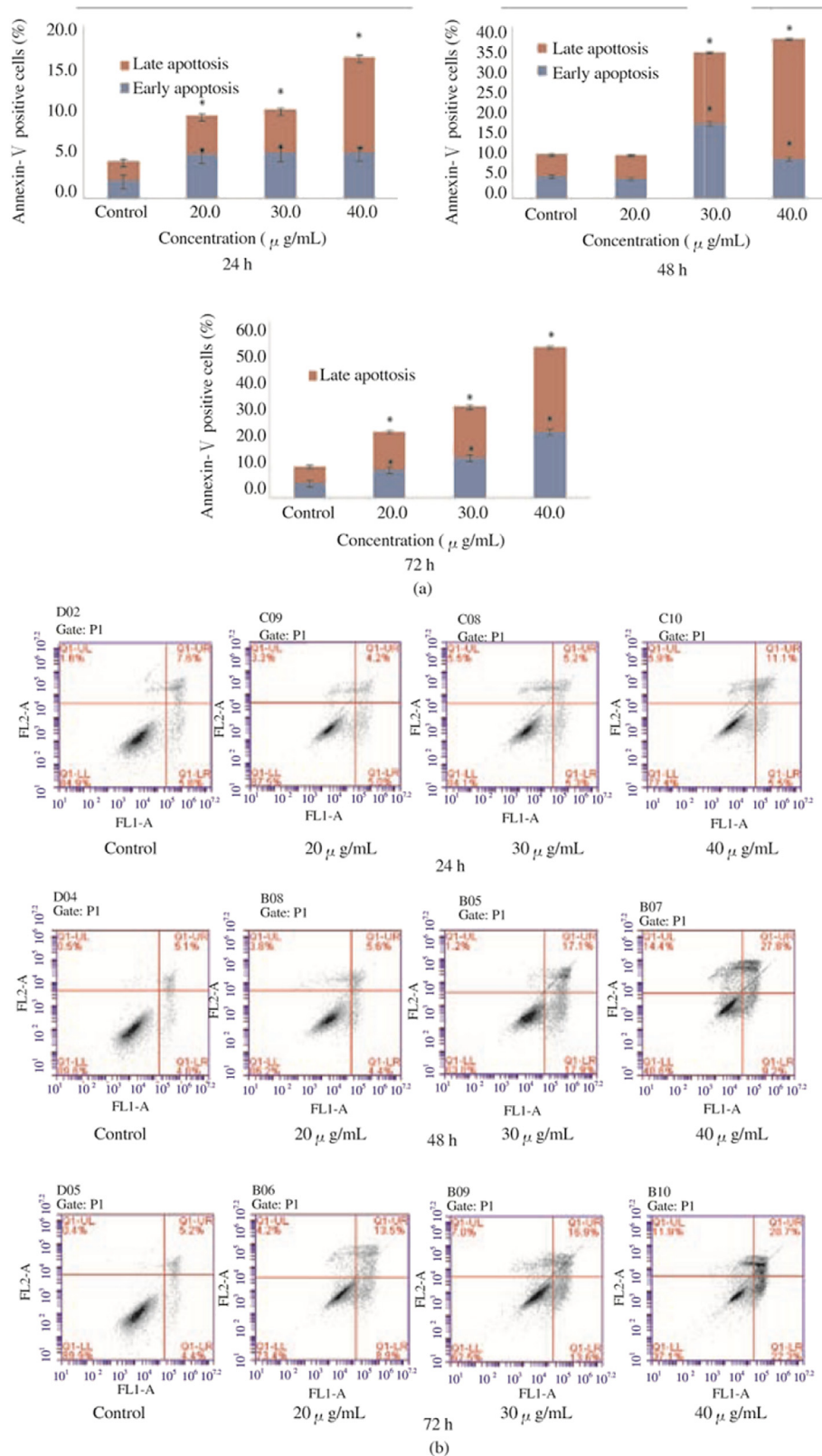
**Figure 5.** Early cell apoptosis detection by Annexin-V/PI staining.

(a) CMH increased apoptotic cells significantly at 20  $\mu$ g/mL, 30  $\mu$ g/mL and 40  $\mu$ g/mL. Compared to the untreated control, treated cells showed statistically significant ( $P < 0.05$ ). An increase in the percentage of apoptotic cells was observed after treatment for 24 h, 48 h, and 72 h. (b) HT29 cells were treated with CMH at 20  $\mu$ g/mL, 30  $\mu$ g/mL, and 40  $\mu$ g/mL for 24 h, 48 h, and 72 h. The lower left quadrant show viable cell population, the lower right quadrant show early apoptotic cell population, the upper right show late apoptotic or necrotic cell and the upper left show necrotic cell.

### 3.3. CMH and CME extracts induced early and late apoptosis in HT29 cells

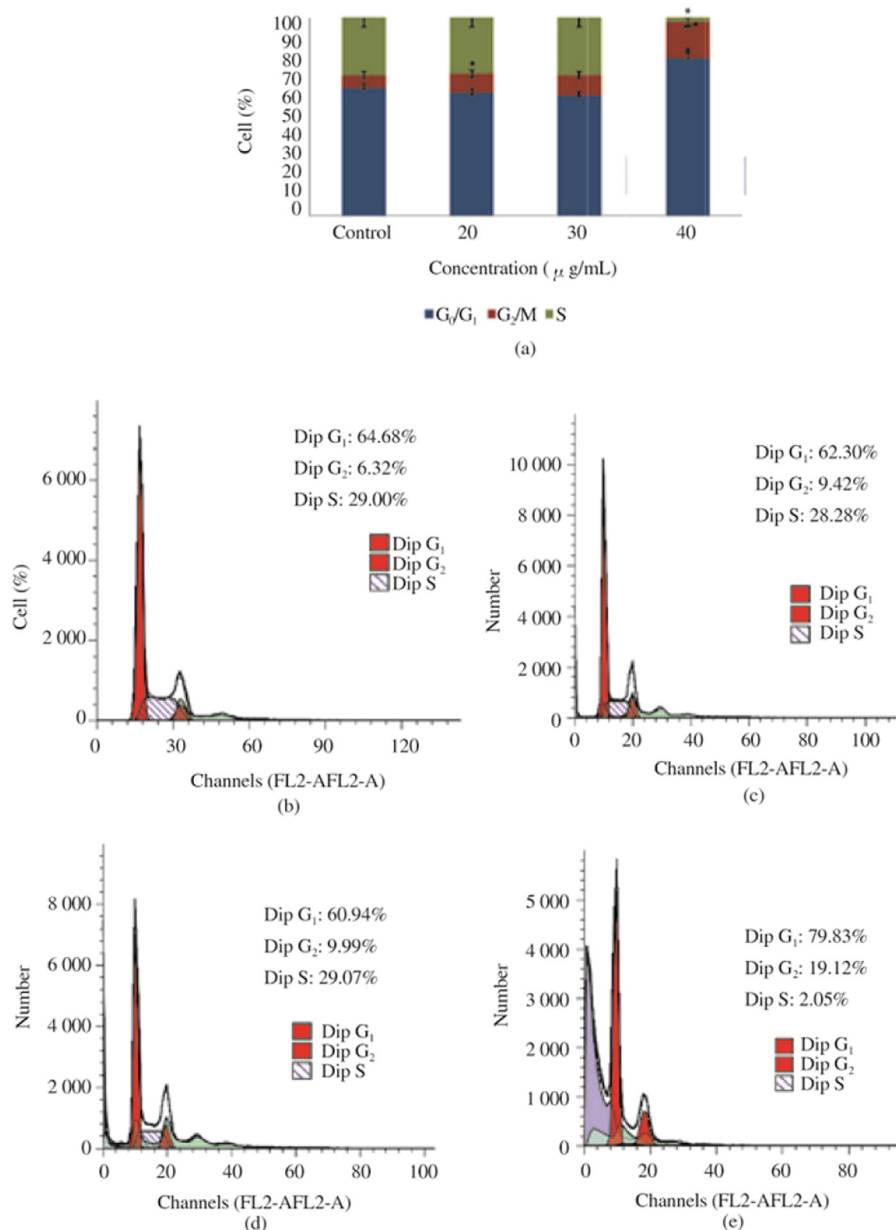
Figures 5 and 6 showed the results of early and late cell apoptosis detection by Annexin-V/PI staining. The extracts induced apoptosis in dose- and time-dependent manner. The population of early apoptotic cells (Annexin-V single positive

cells) increased from  $(6.4 \pm 0.5)\%$  (control) to  $(21.7 \pm 0.3)\%$ , and  $(29.9 \pm 0.5)\%$  for cells treated with 30  $\mu$ g and 40  $\mu$ g/mL. As for CME treated HT29 cells, the population of early apoptotic cells increased from  $(4.9 \pm 0.2)\%$  (control) to  $(9.5 \pm 0.6)\%$ ,  $(13.5 \pm 0.1)\%$ , and  $(22.3 \pm 0.1)\%$  after 72 h treatment with 20, 30 and 40  $\mu$ g/mL of extracts respectively. The population of late apoptotic cells (Annexin-V/PI double positive cells) in CMH



**Figure 6.** Late cell apoptosis detection by Annexin-V/PI staining.

(a) CME has increased the induction of apoptotic cells significantly at 30 µg/mL and 40 µg/mL. Compared to the untreated control, treated cells showed statistically significant ( $P < 0.05$ ). An increase in the percentage of apoptotic cells was observed after treatment for 24 h, 48 h, and 72 h. (b) HT29 cells were treated with CME at 20, 30, and 40 µg/mL for 24, 48, and 72 h. The lower left quadrant show viable cell population, the lower right quadrant show early apoptotic cell population, the upper right show late apoptotic or necrotic cell and the upper left show necrotic cell.



**Figure 7.** Effects of CMH on cell cycle distribution on HT29 cells.

(a) Cells were treated with CMH at different concentrations. Compared to the untreated control, treated cells showed statistically significant, especially after 72 h of treatment ( $P < 0.05$ ). (b) Cell cycle distribution of untreated cells. (c) Cell cycle distribution at 20 µg/mL. (d) Cell cycle distribution at 30 µg/mL. (e) Cell cycle distribution at 40 µg/mL.

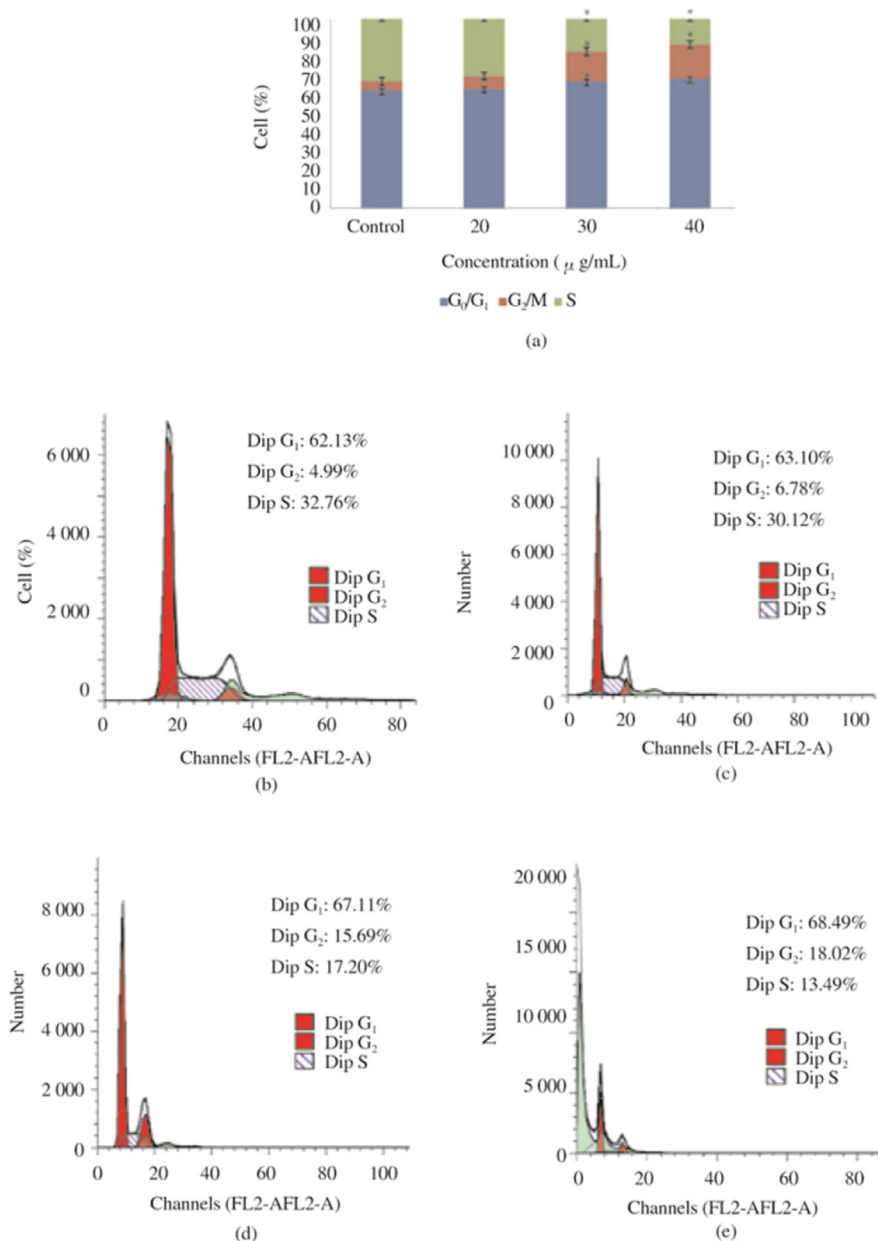
treated cells also increased from  $(7.9 \pm 0.8)\%$  (control) to  $(11.1 \pm 0.3)\%$ ,  $(17.1 \pm 1.3)\%$  and  $(25.8 \pm 0.4)\%$  while in CME treated cells, the population of late apoptotic cells increased from  $(5.6 \pm 0.3)\%$  (control) to  $(13.0 \pm 0.7)\%$ ,  $(17.3 \pm 0.5)\%$  and  $(28.8 \pm 0.5)\%$  after 72 h treatment with 20, 30 and 40 µg/mL of extracts respectively.

### 3.4. Effects of CMH and CME on cell cycle distribution on HT29 cells

CME at concentration 30 and 40 µg/mL and CMH at concentration 40 µg/mL caused accumulation of cells in G<sub>0</sub>/G<sub>1</sub> phase and G<sub>2</sub>/M phase in HT29 cells where the percentages cells in the S phase was significantly reduced (Figures 7 and 8). The

percentages of cells which accumulated in the G<sub>0</sub>/G<sub>1</sub> phase after treatment with CME, increased from  $(62.13 \pm 1.33)\%$  (control) to  $(63.10 \pm 3.35)\%$ ,  $(67.11 \pm 2.51)\%$  and  $(68.49 \pm 1.26)\%$  in 20, 30 and 40 µg/mL respectively. In the G<sub>2</sub>/M phase, the accumulation of cells increased from  $(4.99 \pm 0.91)\%$  (control) to  $(6.78 \pm 1.61)\%$ ,  $(15.69 \pm 1.32)\%$  and  $(18.02 \pm 0.87)\%$  when treated with increasing concentration of CME (20–40 µg/mL). The percentages of cell accumulation in G<sub>0</sub>/G<sub>1</sub> phase treated with CMH increased from  $(67.11 \pm 2.51)\%$  at 30 µg/mL to  $(79.83 \pm 0.46)\%$  at 40 µg/mL. For cells percentage accumulated in G<sub>2</sub>/M phase, the cells increased from  $(6.32 \pm 0.88)\%$  (control),  $(9.42 \pm 2.55)\%$ ,  $(9.99 \pm 3.41)\%$  and  $(18.12 \pm 0.67)\%$  when treated with increasing concentration of CMH (20–40 µg/mL).





**Figure 8.** Effects of CME on cell cycle distribution on HT29 cells.

(a) Cells were treated with CME at different concentrations. Compared to the untreated control, treated cells showed statistically significant, especially after 72 h of treatment ( $P < 0.05$ ). (b) Cell cycle distribution of untreated cells. (c) Cell cycle distribution at 20 µg/mL. (d) Cell cycle distribution at 30 µg/mL. (e) Cell cycle distribution at 40 µg/mL.

#### 4. Discussion

This report attempts to investigate the mechanism of induction of apoptosis induced by ethyl acetate and hexane extracts of *C. mangga* on HT29 cells. The toxicity of both extracts on normal colon cell line, CCD-18Co was evaluated as well. Up to date, this is the first report on the induction of apoptosis by *C. mangga* extracts on HT29 cells.

Both CMH and CME possessed good cytotoxic activity against HT29 cells with IC<sub>50</sub> value of (15.6 ± 0.8) µg/mL and (17.9 ± 1.2) µg/mL respectively in comparison to cisplatin (positive control) with IC<sub>50</sub> value of (8.9 ± 1.2) µg/mL. Dose- and time-dependent cytotoxicity of both extracts on HT29 cells was observed where percentage of cell proliferation inhibition increased as the dose of extracts and exposure periods to extracts

increased. Both extracts possessed mild toxicity on the normal colon cell lines, CCD-18Co, [IC<sub>50</sub> value of (46.5 ± 0.5) µg/mL and (45.7 ± 1.0) µg/mL respectively] in comparison with cisplatin with IC<sub>50</sub> value of (5.5 ± 0.5) µg/mL. Toxicity on the normal tissue of human body is the main problem to the clinical efficacy of chemotherapy [27,28]. Therefore, from the first discovery stages of drug until the mass scale production, one of the biggest requirements in anticancer drug development is the particularly high number of safety precaution in conducting of the drug substance [29].

Phase-contrast microscopy revealed early sign of morphological changes of cell apoptosis such as cell shrinkage, membrane blebbing and formation of apoptotic bodies [20,23,30]. The result showed cell detachment from the bottom surface of culture plate after the cells were exposed to both extracts for 24, 48 and

72 h. Further morphological changes such as DNA condensation and fragmentation were observed under fluorescence which were the characteristics of cell apoptosis [21].

Annexin-V staining was performed to detect the early and late apoptosis in treated HT29 cells. Annexin-V dye can easily bind to the externalized phosphatidylserine on the outer plasma membrane of apoptotic cells [21]. The presence of PS on the cell surface is one of the most significant characteristics in apoptosis due to the negative charge phospholipid with its ability to modify the interactions with other lipids that might disrupts the lipid supporting structures [31]. During occurrences of early apoptosis, the cell membrane asymmetry is disrupted causing the exposure of PS residues to outer cell membrane leaflet [32,33]. Cells in the late apoptosis or secondary necrosis are both Annexin and PI positive. Detection of early and late apoptosis in HT29 cells after treated by CMH and CME are confirmed by positive Annexin-V staining cells in dose- and time dependent manners.

This is further supported by the results from cell cycle analysis where cells were treated with both extracts were arrested in the G<sub>0</sub>/G<sub>1</sub> phase. G<sub>1</sub> phase arrest correlated with the regulation of tumour protein, p53 [34–36] and other proteins such as p21, CDK2 and CDK4. Lin *et al.* (2007) reported that meclizine caused arrest of cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase of human colorectal carcinoma (HCT116 cells) by up-regulating the p53 and p21/CIP1 proteins which in-turn suppressed the activities of CDK2 and CDK4 kinase activities [37]. p53 was recognized as the protein which control G<sub>1</sub> phase which will either induce cell arrest in G<sub>1</sub> phase, or reunite begins with apoptosis [38]. Therefore, arrest in G<sub>1</sub> phase create the chances of cells to enter repair phase or undergoes apoptosis for maintaining tissue homeostasis [39]. Karsono *et al.* (2014) also showed similar effects on cell distribution in prostatic epithelial cells, PC3, where cells arrest occurred in G<sub>0</sub>/G<sub>1</sub> phase when treated with 70% ethanol extract of *C. mangga* [18]. The effect on the cell cycle progression may be contributed by an array of bioactive compounds present in them. Chemical investigations on extracts of *C. mangga* revealed that many of the isolated compounds are labdane-type diterpenoids [7,12,40]. The biosynthetic pathway for labdane-type diterpenoids is selective for this species as diterpenoids are rarely reported present in *Curcuma* species [40].

Based on this study, extracts of *C. mangga* rhizomes displayed inhibitory effects and which able to induce apoptosis on HT29 cell line. Both extracts also caused cell cycle arrest of the HT29 cells in the G<sub>0</sub>/G phase. The mechanism of apoptosis on HT29 cells should be further investigated. However, this study showed that both extracts could be potential candidates in the development of natural products for anticancer therapy.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

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### References

- [1] Fanali C, Lucchetti D, Farina M, Corbi M, Cufino V, Cittadini A, et al. Cancer stem cells in colorectal cancer from pathogenesis to therapy: controversies and perspectives. *World J Gastroenterol* 2014; **20**(4): 923-942.
- [2] Wong MC, Ching JY, Chan VC, Lam TY, Luk AK, Wong SH, et al. Identification of subjects at risk of proximal advanced neoplasia for colorectal cancer screening. *Eur J Cancer (Oxford, England: 1990)* 2015; **51**(1): 37-44.
- [3] Rasool S, Kadla SA, Rasool V, Ganai BA. A comparative overview of general risk factors associated with the incidence of colorectal cancer. *Tumour Biol J Int Soc Oncodevelopmental Biol Med* 2013; **34**(5): 2469-2476.
- [4] Hassan MR, Lim WWD. *The first annual report of the national cancer patient registry-colorectal cancer, 2007–2008*. Kuala Lumpur: National Cancer Patient Registry-Colorectal Cancer and Clinical Research Centre; 2010.
- [5] Ali RM, Samah ZA, Mustapha NM, Hussein N. *ASEAN herbal and medicinal plants*. Jakarta, Indonesia: ASEAN Secretariat; 2010.
- [6] Kirana C, Record IR, McIntosh GH, Jones GP. Screening for antitumor activity of 11 species of Indonesian Zingiberaceae using human MCF-7 and HT-29 cancer cells. *Pharm Biol* 2003; **41**(4): 271-276.
- [7] Malek SNA, Lee GS, Hong SL, Yaacob H, Wahab NA, Faizal Weber JF, et al. Phytochemical and cytotoxic investigations of *Curcuma mangga* rhizomes. *Molecules* 2011; **16**(6): 4539-4548.
- [8] Widowati W, Mozef T, Risdian C, Ratnawati H, Tjahjani S, Sandra F. The comparison of antioxidative and proliferation inhibitor properties of *Piper betle* L., *Catharanthus roseus* [L.] G.Don, *Dendrophoe petandra* L., *Curcuma mangga* Val. extracts on T47D cancer cell line. *Int Res J Biochem Bioinform* 2011; **1**(2): 22-28.
- [9] Widowati W, Mozef T, Risdian C, Yellianty Y. Anticancer and free radical scavenging potency of *Catharanthus roseus*, *Dendrophoe petandra*, *Piper betle* and *Curcuma mangga* extracts in breast cancer cell lines. *Oxid Antioxid Med Sci* 2013; **2**(2): 137-142.
- [10] Abas F, Hui LS, Ahmad S, Stanslas J, Israf DA, Shaari K, et al. Biological evaluation of curcumin and related diarylheptanoids. *Z Naturforsch C – J Bioscie* 2006; **61**(9–10): 625-631.
- [11] Liu Y, Nair M. *Curcuma longa* and *Curcuma mangga* leaves exhibit functional food property. *Food Chem* 2012; **135**(2): 634-640.
- [12] Liu Y, Nair MG. Labdane diterpenes in *Curcuma mangga* rhizomes inhibit lipid peroxidation, cyclooxygenase enzymes and human tumour cell proliferation. *Food Chem* 2011; **124**(2): 527-532.
- [13] Pujimulyani D, Raharjo S, Marsono Y, Santoso U. The effects of blanching treatment on the radical scavenging activity of white saffron (*Curcuma mangga* Val.). *Int Food Res J* 2010; **17**: 615-621.
- [14] Pujimulyani D, Raharjo S, Marsono Y, Santoso U. The effect of blanching on antioxidant activity and glycosides of white saffron (*Curcuma mangga* Val.). *Int Food Res J* 2012; **19**(2): 617-621.
- [15] Pujimulyani D, Raharjo S, Marsono Y, Santoso U. The phenolic substances and antioxidant activity of white saffron (*Curcuma mangga* Val.) as affected by blanching methods. *Int J Biol Vet Agric Food Eng* 2013; **7**: 607-611.
- [16] Ruangsang P, Tewtrakul S, Reanmongkol W. Evaluation of the analgesic and anti-inflammatory activities of *Curcuma mangga* Val and Zipp rhizomes. *J Nat Med* 2010; **64**(1): 36-41.
- [17] Philip K, Malek SN, Sani W, Sim KS, Kumar S, Hong SL, et al. Antimicrobial activity of some medicinal plants from Malaysia. *Am J Appl Sci* 2009; **6**(8): 1613-1617.
- [18] Karsono AH, Tandrasasmita OM, Tjandrawinata RR. Molecular effects of bioactive fraction of *Curcuma mangga* (DLBS4847) as a downregulator of  $\alpha$ -reductase activity pathways in prostatic epithelial cells. *Cancer Manag Res* 2014; **6**: 267-278.
- [19] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**(5): 646-674.
- [20] Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res CR* 2011; **30**: 87.

- [21] Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007; **35**(4): 495-516.
- [22] Lawen A. Apoptosis-an introduction. *Bio Essays News Rev Mol Cell Dev Biol* 2003; **25**(9): 888-896.
- [23] Hung RWY, Chow AW. Apoptosis: molecular mechanisms, regulation and role in pathogenesis. *Can J Infect Dis* 1997; **8**(2): 103-109.
- [24] Houghton P, Fang R, Techatanawat I, Steventon G, Hylands PJ, Lee CC. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods (San Diego, Calif)* 2007; **42**(4): 377-387.
- [25] Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Nat Cancer Inst* 1991; **83**(11): 757-766.
- [26] Hubbard KS, Gut IM, Scheeler SM, Lyman ME, McNutt PM. Compatibility of SYTO 13 and Hoechst 33342 for longitudinal imaging of neuron viability and cell death. *BMC Res Notes* 2012; **5**: 437.
- [27] Ho YF, Karsani SA, Yong WK, Abd Malek SN. Induction of apoptosis and cell cycle blockade by helichrysetin in A549 human lung adenocarcinoma cells. *Evid Based Complement Alternat Med* 2013; **2013**: 10.
- [28] Remesh A. Toxicities of anticancer drugs and its management. *Int J Basic Clin Pharmacol* 2012; **1**(1): 2-12.
- [29] Narang AS, Desai DS. *Anticancer drug development. Pharmaceutical perspectives of cancer therapeutics*. New York: Springer; 2009, p. 49-92.
- [30] Ziegler U, Groscurth P. Morphological features of cell death. *Physiology* 2004; **19**(3): 124-128.
- [31] Demchenko AP. The change of cellular membranes on apoptosis: fluorescence detection. *Exp Oncol* 2012; **34**(3): 263-268.
- [32] van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998; **31**(1): 1-9.
- [33] van Engeland M, Kuijpers HJ, Ramaekers FC, Reutelingsperger CP, Schutte B. Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp Cell Res* 1997; **235**(2): 421-430.
- [34] Kastan MB, Kuerbitz SJ. Control of G1 arrest after DNA damage. *Environ Health Perspect* 1993; **101**(Suppl 5): 55-58.
- [35] Bartek J, Lukas J. Mammalian G<sub>1</sub>- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol* 2001; **13**(6): 738-747.
- [36] Pucci B, Kasten M, Giordano A. Cell cycle and apoptosis. *Neoplasia (New York)* 2000; **2**(4): 291-299.
- [37] Lin JC, Ho YS, Lee JJ, Liu CL, Yang TL, Wu CH. Induction of apoptosis and cell-cycle arrest in human colon cancer cells by meclizine. *Food Chem Toxicol* 2007; **45**(6): 935-944.
- [38] Kagawa S, Fujiwara T, Hizuta A, Yasuda T, Zhang W-W, Roth JA, et al. p53 expression overcomes p21WAF1/CIP1-mediated G<sub>1</sub> arrest and induces apoptosis in human cancer cells. *Oncogene* 1997; **15**(16): 1903-1909.
- [39] Wang Y, Zhou Y, Zhou H, Jia G, Liu J, Han B, et al. Pristimerin causes G<sub>1</sub> arrest, induces apoptosis, and enhances the chemosensitivity to gemcitabine in pancreatic cancer cells. *PLoS One* 2012; **7**(8): e43826.
- [40] Abas F, Lajis NH, Shaari K, Israf DA, Stanslas J, Yusuf UK, et al. A labdane diterpene glucoside from the rhizomes of *Curcuma mangga*. *J Nat Prod* 2005; **68**(7): 1090-1093.